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Thanks.

Overexpression of Lerk-5/Eplg5 Messenger RNA: A Novel Marker for Increased Tumorigenicity and Metastatic Potential in Human Malignant Melanomas[1]

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ABSTRACT

The Lerks, ligands of eph-related receptor tyrosine kinases, are a rapidly expanding family of genes thought to play an important role in the development and oncogenesis of various tissues. However, very little experimental evidence supports this hypothesis. Using RNA fingerprinting, we detected increased expression of Lerk-5 mRNA in human melanocytes as a response to the tumor-promoting drug 12-*O*-tetradecanoylphorbol-13-acetate, which suggests a possible role of the Lerks in melanoma tumorigenesis and progression. Therefore, we studied Lerk-5 mRNA expression in various melanoma cell lines and tissues of melanocytic tumors by semiquantitative reverse transcription-PCR. Modest expression of Lerk-5 mRNA was found in two melanoma cell lines derived from early primary tumors (WM35 and WM1645B); two metastatic cell lines tested showed a 3.9-fold increased transcript abundance when compared to the primary cell lines (RPMI-7951 and SK-Mel5). Progeny of a melanoma cell line with very low Lerk-5 mRNA abundance (WM35) showed a 5-fold increase in Lerk-5 mRNA expression when it was selected for higher tumorigenicity and multicytokine resistance by passaging in nude mice or repeated high-dose UVB irradiation. Consistent with these experimental data, we found high levels of Lerk-5 mRNA expression in advanced primary malignant melanomas and metastases ($n = 22$) but significantly lower or undetectable mRNA expression in benign melanocytic nevi ($n = 9$; $P < 0.001$). We conclude that increased Lerk-5 expression possibly reflects or induces an increased potential of growth, tumorigenicity, and metastatic abilities in human melanomas. This makes the yet to be elucidated eph-related receptor tyrosine kinase/Lerk signaling system a potential new source for molecular markers as well as a target for new therapies.

INTRODUCTION

Systematic cDNA scanning experiments of human melanocytes that used the RAP-PCR[3] (1) led to the detection of more than a dozen new expressed sequence tags and of several known mRNAs that were not known to be expressed in melanocytes (2). Among these, the expression of Lerk5 mRNA is of particular interest because a pivotal role of the eph-RTK/Lerk signaling system was postulated in differentiation and, possibly, in oncogenesis of various tissues, particularly in tissues of neuroectodermal origin (3–7). Recently, Lerk-1/protein B61 was shown to stimulate the growth of eck-expressing melanoma cell lines but not normal cultured melanocytes, suggesting that Lerk-1 may be an autocrine growth factor in malignant melanomas (8).

Lerk-5/Eplg5, also referred as Elf2/HTK-L, is a member of a family of ligands of the eph-RTKs (9, 10). At least seven different ligands that share close amino acid identities, ranging from 28 to 59%, have been described (3, 11). Lerk-5 is predicted to be a type 1 transmembrane protein that is most similar to Lerk-2 (4, 12) and Elk-L3/NLerk-2 (13). Binding of these membrane-bound ligands requires cell-to-cell contact (14) and can induce phosphorylation of the eph-RTKs hek, elk, and htk (10). All three ligands share strong sequence conservation of their cytoplasmic domains, including five invariant tyrosine residues, which can become phosphorylated themselves, suggesting a dual role for this type of transmembrane eph-RTK ligand (5): they can probably activate signaling pathways in receptor-expressing cells, and in addition, their cytoplasmic domains may function as substrates of tyrosine kinases (*e.g.*, by platelet-derived growth factor receptor tyrosine kinase), allowing interactions with other proteins that may activate signaling pathways in ligand-expressing cells (5).

This study provides the first description of a functional context in which Lerk-5 may be involved and suggests a possible role of Lerk-5 in melanoma tumorigenesis and progression. We analyzed the expression patterns of Lerk-5 mRNA in a

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[3] The abbreviations used are: RAP-PCR, RNA arbitrarily primed PCR; eph-RTK, eph-related receptor tyrosine kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SSCP, single-stranded conformation polymorphism; RT-PCR, reverse transcription-PCR; RALT, relative abundance of Lerk-5 transcript; PKC, protein kinase C.

series of melanoma cell lines under various experimental conditions and tissues of melanocytic tumors. Our data indicate that increasing dedifferentiation and metastatic potential of malignant melanoma are paralleled by up-regulation of Lerk-5 mRNA expression. Therefore, potential clinical applications of this novel molecule as a prognostic and diagnostic marker can be envisioned. Furthermore, we could confirm the location of this gene on chromosome 13q33 and add further sequence information to the current GenBank database entry (new GenBank accession no. U81262).

MATERIALS AND METHODS

Cell Culture and Tissue Samples. Cryopreserved human Caucasian newborn melanocytes were purchased from Clonetics Corp. (San Diego, CA) and grown in a MCDB153 formulation, supplemented with 1 ng/ml human basic fibroblast growth factor, 0.5 μg/ml hydrocortisone, 10 ng/ml (16 nM) TPA, 5 μg/ml insulin, and 15 μg/ml bovine pituitary extract. Melanoma cell lines derived from early primary melanomas were WM35 and WM1341B, and some WM35 cells were selected for enhanced tumorigenicity and multicytokine resistance by passage in nude mice; these are referred to as 35-P2-N1 and 35-P2-N2, respectively (15). In addition, two fast growing cell lines from lymph node metastases of malignant melanomas, HTB66/RPMI-7951 and HTB70/SK-MEL-5, were purchased from American Type Culture Collection (Rockville, MD). All melanoma cells were grown in a RPMI 1640 formulation (Life Technologies, Inc., Grand Island, NY) supplemented with 5% FCS. Tissues from melanocytic tumors were collected at the Department of Dermatology, University of Regensburg (Regensburg, Germany). Written consent of the patients was obtained before the operations took place. Diagnoses of the tissues analyzed were confirmed by two expert histopathologists prior to RNA extraction (W. S. and M. L.). As a UV source for UV resistance selection, we used a customized apparatus (Stratagene, San Diego, CA) with a built-in flux measurement device. The UV lamps provided by the manufacturer had a continuous spectrum of UVB light (290–320 nm) with a peak at 312 nm. The decrease of emission energy with shorter wavelengths is similar to the pattern observed with the sun, so the emission in the UVC range (<290 nm) is negligible. About 15% of the total energy is emitted in the UVA range (320–400 nm). Mean UVB flux rates were 20 J/s.

RAP-PCR and Isolation of Differentially Amplified Transcripts. Total cellular RNA was extracted using the RNeasy spin column purification kit (Qiagen, Chatsworth, CA). Cultured cells were directly lysed by addition of lysis buffer to the culture dish, whereas tissues required microdissection prior to homogenization. Pieces of tumor-carrying skin were snap frozen after removal and stored at −80°C. After establishment of the diagnoses by expert histopathologists (W. S. and M. L.), based on sections and routine staining of paraffin-embedded parts of the same tumor, 10-μm sections of the frozen pieces were cut and stained to localize the tumor within the skin sample. Subsequently, thick sections (up to 1 mm) were dissected using microscope binoculars. Contaminating parts of epidermis, the dermis, and the s.c. fat were removed. The remaining pieces were collected into a cell lysis buffer (Qiagen, Chatsworth, CA) and homogenized with glass pestels. Finally, another 10-μm section was taken from the remaining tissue and stained as an additional control for correct dissection of the tumor nests. The Qia-shredder columns were used to further homogenate the lysates and to break down genomic DNA (Qiagen, Chatsworth, CA). To eliminate the remaining genomic DNA, the total extracted RNA was treated with DNase I as described (1). RAP-PCR of total cellular RNA was performed as described previously (1). Arbitrary primers that produced the fingerprint, from which the Lerk-5 cDNA fragment was isolated, were "AP-11" (5′-AGGGGCACCA-3′) for first strand synthesis and "AP-4" (5′-GCACCAGGGG-3′) for second strand synthesis. The RNA fingerprint is shown in Fig. 1*A*. This is one of six fingerprints generated using these RNAs and various arbitrary primers. Isolation of putatively differentially amplified RAP-PCR products was achieved by purification of the desired product with native SSCP gels as described (16). Cloning and sequencing of the selected cDNAs followed standard procedures.

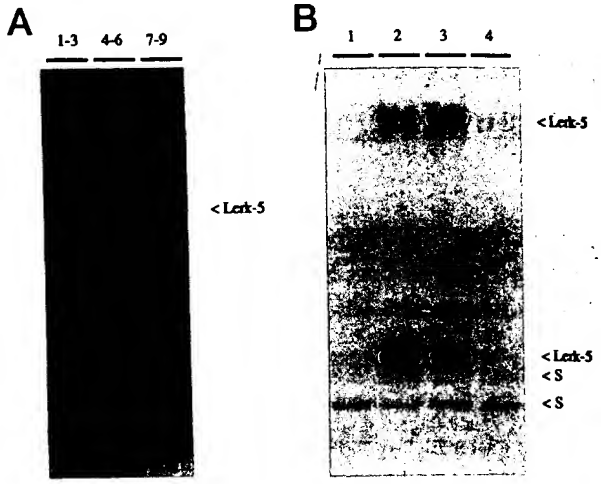

Fig. 1 RAP-PCR fingerprinting reveals up-regulation of Lerk-5 mRNA in TPA-treated normal human melanocytes. *A*, starved normal human newborn melanocytes (*Lanes 1–3*) were treated with the tumor-promoting drug TPA (32 nM; *Lanes 7–9*) and with TPA in combination with 0.02 mg/ml cycloheximide (*Lanes 4–6*). Eight h after the treatments, total RNA was prepared and fingerprinted at three 2-fold dilutions (400, 200, and 100 ng of total RNA) using the arbitrary primers AP-11 (5′-AGGGGCACCA-3′) and AP-4 (5′-GCACCAGGGG-3′). The RAP-PCR products were loaded side by side so that each set of three lanes represented three RNA concentrations of each treatment group and were electrophoresed on an 8 M urea/6% polyacrylamide gel. *Arrowhead*, band from which we obtained the Lerk-5 cDNA fragment. *B*, differential expression of the Lerk-5 mRNA transcript (*arrowhead, Lerk-5*), dependent on TPA treatment, was then confirmed by relative, semiquantitative RT-PCR using a piece of 18S rRNA as internal standard (*arrowhead, S*). *Lane 1*, starved normal human melanocytes; *Lanes 2* and *3*, melanocytes treated with 32 nM TPA for 4 and 24 h; *Lane 4*, melanocytes treated with 32 nM TPA for 4 h and simultaneously growth arrested by a single UVB treatment (2000 J/m^2). RT-PCR products were electrophoresed on a nondenaturing (SSCP) gel for 24 h. Therefore, Lerk-5 mRNA and internal standard are both represented by two bands. Quantitation was performed by β-counting of the upper band, corresponding to Lerk-5 mRNA abundance relative to the lower band of the internal 18S rRNA standard, termed RALT.

Confirmation of Differential Gene Expression by Semiquantitative, Relative RT-PCR. Due to the analysis of small tissue samples with low yield of RNA, we used semiquantitative, relative RT-PCR (QuantumRNA module; Ambion, Austin, TX). The QuantumRNA module provides a method for comparing relative transcript abundances standardized by coamplification of a highly conserved fragment of 18S rRNA as an invariant internal standard. This is only possible if both the target transcript and the reference are analyzed while PCR is still in exponential phase of amplification. Pilot experiments were performed to determine these conditions for the fragment of the Lerk-5 mRNA transcript that was amplified. The amplification efficiency of the coamplifying 18S RNA standard was then adjusted to the efficiency of the amplification of the target transcript by the addition of increasing amounts of competitor primers (Competimers), *i.e.*, 18S rRNA primers that are modified at their 3′ ends to block extension by DNA polymerase. The cycling of both the target and the standard was performed in a single tube. For Lerk-5 cDNA amplification, the primers 5′-TAA AGA CCA AGC AGA CAC AGA TGC AC-3′ and 5′-TAC TTC AGC AAG AGG ACC ACC AGC-3′ were used, which produce a 510-bp product from the coding sequence of Lerk-5 mRNA. cDNAs were produced by the RT-System (Promega, Madison, WI), starting with 1 μg of total RNA. The 10-fold diluted cDNA solutions (1.5 μl) were added to 10 μl of 2× PCR mixture [2 units/reaction AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT), 2× PCR buffer II, 4 mM $MgCl_2$, 0.4 mM each dNTP, 2 μCi/reaction [α-^{32}P]dCTP, 0.75 μM primer mixture, and 1.6 μl of standard primer mixture with Competimer:18S rRNA primer at 8:2] and cycled through 20 cycles (94°C, 30 s; 66°C, 30 s; 72°C, 1 min). Three μl of the complete reaction were mixed with 12 μl of formamide dye buffer and denatured at 95°C for 2 min. Two μl of this solution were loaded onto a nondenaturing 6% polyacrylamide gel for maximum resolution (SSCP gel). Electrophoresis was performed for 24 h at 5 W in 0.6× Tris-borate EDTA buffer. Gels were then transferred to 3-mm Whatman paper and dried under vacuum at 80°C. Bands of the Lerk product and the internal standard were subjected to β-counting and subsequent data analysis using the Ambis software (Ambis Inc., San Diego, CA). RALT was calculated as the ratio of the counts of the upper Lerk-5 band (510 nucleotides) and the lower band of the internal 18S RNA standard (488 nucleotides), as shown in Fig. 1*B*. Differences between different tissues were tested for significance using the Mann-Whitney *U* test.

RESULTS

Characterization of Parts of the 3′ Untranslated Region of the Human Lerk-5 mRNA Transcript. Total RNAs prepared from normal human melanocytes treated with TPA after starvation were surveyed by RAP-PCR. Fig. 1*A* shows the RNA fingerprint from which the Lerk-5 cDNA fragment was isolated. The fingerprint indicated a >4-fold increased amplification of this fragment in the TPA-treated group *versus* the TPA-starved group. Database analysis of the sequence of this fragment was performed on-line using blastn/nr at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). The sequence was found to be 100% homologous, to GenBank accession no. HUMCH13GEN between bases 1007 and 1157, which is known to map to chromosome 13q33. In addition, it showed significant homology to 3′ untranslated regions of the Lerk-5 mRNA sequence in mouse (GenBank accession no. MMU16819) and the hepatoma transmembrane kinase ligand in mouse, HTK (GenBank accession no. MUSHTK). The HUMCH13GEN clone has a 294-base overlap with the entry for human Lerk-5 mRNA (GenBank accession no. HSU16797), as well as a 435-base overlap with the human hepatoma transmembrane kinase ligand gene (GenBank accession no. HUMHTK), which is identical to Lerk-5 (10). Thus, we can confirm the location of the human Lerk-5 gene on chromosome 13q33, which was previously anticipated by interspecific back-cross analysis (10). By fusion of the HUMCH13GEN clone sequence with the previous entry for the human Lerk-5 cDNA sequence, the available sequence information of the Lerk-5 mRNA transcript was expanded to a total of 2902 bases (new GenBank accession no. U81262).

Table 1 Differential Lerk-5 mRNA expression in normal human melanocytes and malignant melanoma cell lines

Cell line	Treatment	RALT[a]	Regulative pattern[b]
Normal melanocytes	No TPA	0.19	+
	TPA, 4 h	1.99	++++
	TPA, 24 h	2.26	+++++
	TPA/UVB, 4 h	0.62	++
WM1645B		0.56	++
HTB66		1.44	+++
HTB70		1.19	+++
WM35		0.11	+
	Passaged in nude mice	0.55	++
	UVB	0.68	++
	TPA, 4 h	2.11	+++++
	TPA, 24 h	0.48	+

[a] RALT was defined as the ratio of the counts of the Lerk-5 mRNA PCR product and 18S rRNA internal standard PCR product on the gels.
[b] Relative regulation in comparison to quiescent normal melanocyte: +, 0.00–0.49; ++, 0.50–0.99; +++, 1.00–1.49; ++++, 1.50–1.99; +++++, >2.00.

Lerk-5 mRNA Expression in Normal Human Melanocytes Reflects Growth Stimulation by TPA. A tissue culture experiment was designed to investigate Lerk-5 transcript abundance modulated by TPA. TPA induces an activated status of normal quiescent melanocytes, characterized by adoption of tumor-like features, most importantly, rapid growth cycling. However, growth remains limited to about 15–20 generations of cells (17–19). TPA is commonly used as a supplement of culture medium to study melanocytes, which otherwise quickly become senescent and die (20, 21). In this experiment, the cells received fresh medium each day during days 1–6. On day 7, all cells received TPA-free medium for a period of 20 h, a time that induces G_0 in melanocytes (19, 21). On day 8, the cells got fresh medium, either supplemented with TPA (32 nM) or not. The TPA dose used was within a broad, dose range (10–100 nM) that uniformly activates growth of human melanocytes, probably by stimulating different PKC isoforms and modulating their cellular location (19, 21). Furthermore, a fraction of cells was G_1 arrested by irradiation with a previously determined dose of

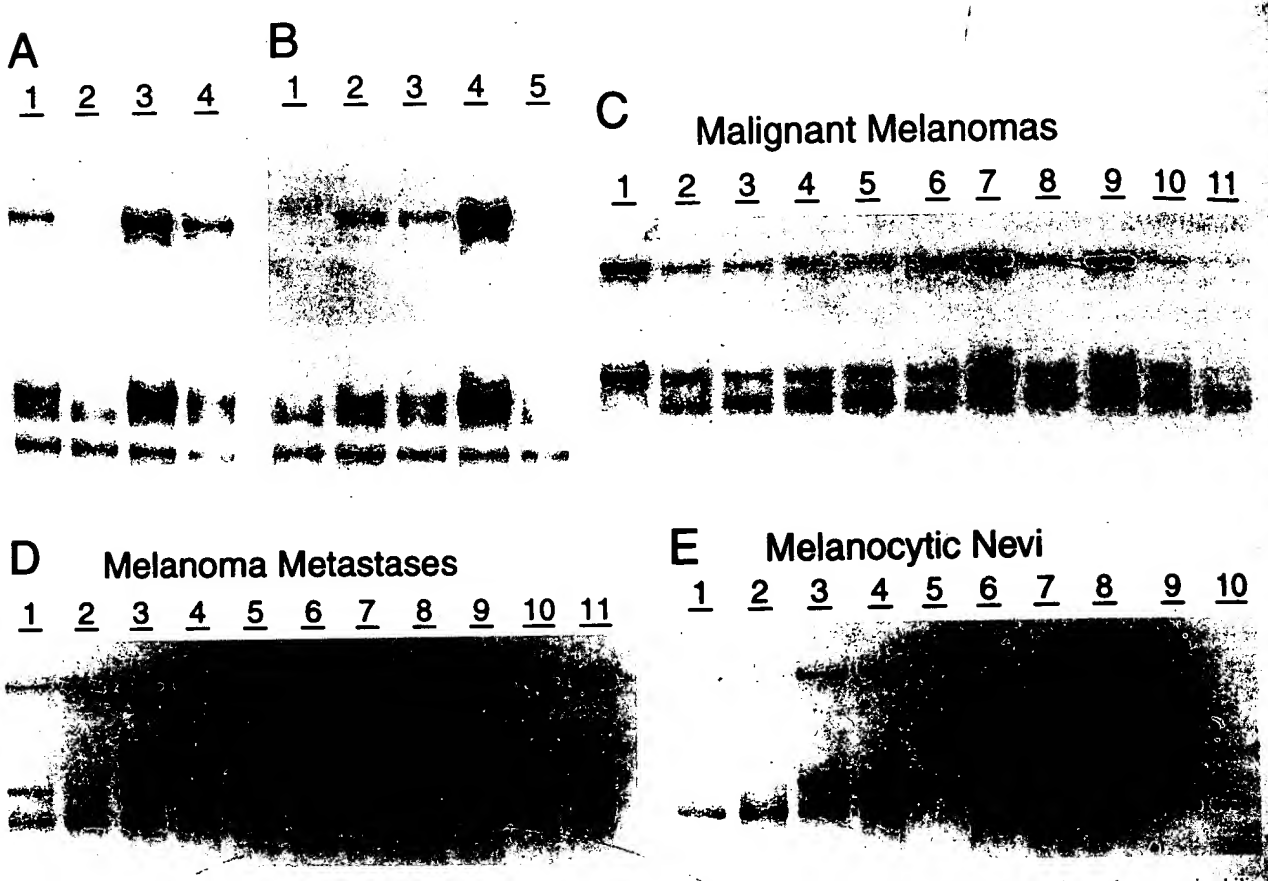

Fig. 2 Relative semiquantitative RT-PCR confirms Lerk-5 mRNA expression as a putative indicator of higher tumorigenicity and metastatic ability. *A*, Lerk-5 mRNA expression in human melanoma cell lines. *Lanes 1* and *2*, cell lines derived from initial malignant melanomas (WM1645B and WM35); *Lanes 3* and *4*, metastatic cell lines (RPMI-7951 and SK-Mel5). *B*, increased expression of Lerk-5 mRNA in WM35 cells (*Lane 1*) selected for higher tumorigenicity by passage in nude mice (*Lane 2*) or selected for UVB damage resistance (*Lane 3*). Short-time treatment with TPA (*Lane 4*, 4 h) also increased Lerk-5 mRNA abundance, whereas long-time treatment (*Lane 5*, 24 h) led to minor up-regulation in this cell line. Up-regulation of Lerk-5 mRNA abundance in advanced malignant melanomas (*C*) and melanoma metastases (*D*) in comparison to melanocytic nevi (*E*; *Lane 5* was skipped). Quantitation was performed by β-counting of the upper band corresponding to Lerk-5 mRNA abundance relative to the lower band of the internal 18S rRNA standard, termed RALT.

UVB (2000 J/m^2) and subsequently treated with 32 nM TPA. Cells from all treatment groups were harvested 4 h after treatment; a second harvest was done in the TPA-treated group after 24 h to determine how transcript abundances after long-term TPA treatment compare to more immediate changes.

By relative RT-PCR, we confirmed TPA inducibility of the Lerk-5 transcript, as predicted by RAP-PCR. The RALT in quiescent cells after 20 h of TPA starvation was 0.19. A 10-fold increase was found in cells that were exposed to TPA for 4 h (RALT = 1.99), and a similarly high transcript abundance was observed after 24 h of TPA treatment (RALT = 2.26). However, the RALT showed only a moderate increase when cells were treated with TPA and simultaneously arrested in G_1 by UVB irradiation, suggesting a correlation of Lerk-5 mRNA abundance and melanocytic growth (Fig. 1*B*). Table 1 summarizes the results.

Expression of Lerk-5 mRNA in Cultured Malignant Melanoma Cell Lines. Endogenous Lerk-5 transcript abundance was studied in various malignant melanoma cell lines. RNAs were harvested from cells in exponential growth phase at about 70% confluence. Constitutive endogenous expression of Lerk-5 mRNA was found in two melanoma cell lines derived from early primary tumors (WM35 and WM1645; mean RALT = 0.33). The lowest Lerk-5 mRNA expression was found in the cell line WM35 (RALT = 0.11; Fig. 2*A*). The two more rapidly growing (data not shown) metastatic cell lines tested showed a 4-fold increase of the RALT ratios when compared with the primary cell lines (RPMI-7951 and SK-Mel5; mean RALT = 1.32). The cell line with very low constitutive Lerk-5 mRNA expression, WM35, is derived from an early (thin) primary melanoma (15). Variant sublines of these parentals were produced that were passaged in nude mice. The progeny is known to be highly tumorigenic, *i.e.*, tumorigenic in nude mice without coinjection of Matrigel, and multicytokine resistant (15). In the passaged cells, a 5-fold increase of Lerk-5 mRNA transcript abundance was found (RALT = 0.55). Because UVB irradiation is also known to increase the aggressiveness of human malignant melanomas for growth and metastasis

Table 2 Lerk-5 expression in human melanocytic tumors does not correlate with common prognostic features

Patient initials	Histopathology	Lerk-5 expression (RALT)	Tumor thickness	Clark level	Prognostic index[a]
RT	Melanocytic nevus	0.02			
LP	Melanocytic nevus	0.02			
PT	Melanocytic nevus	0.02			
AA	Melanocytic nevus	0.03			
LE	Melanocytic nevus	0.03			
BG	Melanocytic nevus	0.28			
GC	Congenital melanocytic nevus	0.33			
MP	Congenital melanocytic nevus	0.53			
AF	Congenital melanocytic nevus	0.59			
LL	Nodular melanoma	0.28	1.50	III	5
TA	Nodular melanoma	0.55	4.80	V	78
ST	Nodular melanoma	0.56	1.90	IV	21
GS	Lentigo maligna melanoma	0.62	5.00	V	10
EA	Nodular melanoma	0.65	6.00	V	60
KF	Acrolentiginous melanoma	0.70	3.60	IV	22
MK	Secondary nodular melanoma	0.77	0.70	II	<1
BC	Secondary nodular melanoma	0.82	1.80	III	2
SA	Superficial spreading melanoma	1.01	1.50	IV	ND[b]
GF	Nodular melanoma	1.44	2.30	IV	12
KH	Nodular melanoma	1.67	3.10	V	54
SH	Melanoma metastasis	0.48			
LH	Melanoma metastasis	0.55			
LA	Melanoma metastasis	0.56			
FD	Melanoma metastasis	0.64			
LK	Melanoma metastasis	0.66			
WO	Melanoma metastasis	0.70			
SN	Melanoma metastasis	0.72			
SD	Melanoma metastasis	0.80			
TR	Melanoma metastasis	0.88			
MH	Melanoma metastasis	0.96			
VB	Melanoma metastasis	0.98			

[a] Prognostic index = tumor thickness multiplied by mitotic counts.
[b] ND, not determined.

due to fundamental changes in long-term gene expression (22), we irradiated the cell line WM35 repeatedly with sublethal doses of UVB. Parentals were exposed to 3500 J/m^2 when they reached full confluence, *i.e.*, at a time point when DNA synthesis is declining due to confluence. Cells that survived for >72 h were isolated, and multiple progeny clones were subcultured. This procedure was repeated two more times, with doses increased to 4000 and 4500 J/m^2, respectively. The progeny that tolerated doses of 3500 J/m^2 were regrown until they reached 70% confluence, and then they were harvested. In the corresponding RNAs, similar to the highly tumorigenic nude mice progeny of WM35, a stable Lerk-5 mRNA positive phenotype was found (RALT = 0.68). In a further experiment, we exposed parentals of WM35 to medium that contained 32 nM TPA. RNAs were harvested after 4 and 24 h. Similar to nontransformed cells, a 5.3-fold increase of the relative Lerk-5 mRNA transcript abundance was found after 4 h (RALT = 2.11), whereas the 24-h exposure was associated with only moderate expression of Lerk-5 mRNA (RALT = 0.28). These data are in accordance with the concept of a biphasic response of malignant melanoma cell lines to TPA. A response mode of initial stimulation of growth and metastatic capability and subsequent inhibition has been repeatedly observed (19, 20). Fig. 2*B*, shows the autoradiography of the corresponding SSCP gel. Table 1 summarizes the results of the cell culture experiments.

Lerk-5 mRNA Expression Was Significantly Increased in Malignant Melanomas and Their Metastases in Comparison with Melanocytic Nevi. Bearing in mind the caveats of cell culture experiments, *e.g.*, the presence of artificially added growth factors such as calf serum, basic fibroblast/growth factor, and insulin, we assessed Lerk-5 expression in human melanocytic tumor tissues. The availability of sufficient tissues from melanocytic nevi and primary melanomas is limited due to the need to prepare the tissue almost *in toto* for pathological evaluation of diagnosis and prognosis. Therefore, we sought to determine Lerk-5 mRNA expression in tissues by relative RT-PCR. Consistent with our cell culture experiments, we found very low Lerk-5 mRNA expression in the common melanocytic nevi, 0.06 ± 0.05 (mean RALT ± SD) but significantly increased levels in advanced malignant melanomas and their metastases (0.82 ± 0.40 and 0.72 ± 0.16, respectively; *U* test for nevi *versus* melanomas and *versus* metastases, $P < 0.001$). In adjacent skin (dermis and epidermis), the RALT values were between 0.2 and 0.25, which argues for correct microdissection of the common nevi. Figs. 2, *C–E*, shows the autoradiographs of the corresponding SSCP gels. Table 2 gives the detailed results. Higher amounts of Lerk-5 mRNA were also measurable in the large congenital nevi analyzed, which may indicate a special biology of congenital nevi. In primary melanomas, no correlation was found between RALT values and classical prognostic

features, such as tumor thickness, Clark level of invasion, or prognostic index. Table 2 summarizes the results obtained from tissues of melanocytic tumors.

DISCUSSION

Here, we provide the first experimental evidence that the level of expression of the transcript of the Lerk-5 gene correlates with increased growth, tumorigenicity, and metastatic ability in malignant melanomas. (*a*) We found constitutive overexpression of Lerk-5 mRNA in all of the four malignant melanoma cell lines tested, most prominently in the rapidly growing metastatic ones. (*b*) In addition, selection of melanoma cells for higher tumorigenicity and multicytokine resistance by passage in nude mice (15) was accompanied by a 5-fold increase in Lerk-5 mRNA expression. (*c*) Lerk-5 mRNA overexpression was also a consistent finding in malignant melanomas of the skin and melanoma metastases as compared to common melanocytic nevi. The highest relative expression within the benign tumors was found in large congenital nevi, which may indicate a special biology of this tumor entity. Therefore, Lerk-5 mRNA overexpression has the potential to be used as a new molecular marker for differential diagnosis of malignant melanomas and common nevi, which is a frequent problem in histopathology. In addition, assessment of Lerk-5 mRNA expression is a candidate molecular marker with prognostic significance in stage I malignant melanomas.

The widespread expression pattern of both RTKs and their ligands and the complexity of ligand-receptor interactions with extensive cross-binding of Lerks to the eph-RTKs make defining the specifics of the involved signal transduction pathways a difficult task (9). The discovery of a series of eph-RTK ligands such as Lerk-1/B61 for the receptor eck, Lerk-2 for elk (11), and Lerk-5 for hek, elk, and htk (10) have not yet led to a clear demonstration of the biological effects in response to receptor activation. However, recent data concerning the significance of Lerks for brain development and neuronal pathfinding indicate a crucial importance of this system regarding cell-to-cell interaction among neuroectodermal cells (5). The observations that Lerk-1/B61 is expressed in melanoma cell lines (8) and that Lerk-3/EHK-L/EFL-2 is exclusively expressed in the central nervous system and the skin (14), as well as our observations of Lerk-5 as an indicator of high tumorigenicity and metastatic potential in malignant melanomas, imply important functions in the development and oncogenesis of the skin, particularly of the melanocytic, neural crest-derived system.

Interestingly, in our cell culture experiments, Lerk-5 mRNA expression could consistently be induced by TPA in both melanocytes and cells derived from early nonmetastatic melanoma cells. Phorbol esters, such as TPA, have long been known as very strong tumor-promoting drugs (23). In cell culture, it has been recognized that TPA induces a phenocopy of the malignant phenotype in benign cells (19). As a result, the cells take on signs of changed differentiation and growth habits of their malignant counterparts, which is paralleled by fundamental changes in gene transcription. Accordingly, TPA induces the transition from a quiescent phenotype to a phenotype characterized by tumor-like growth in cultured newborn melanocytes (20, 21). Therefore, as our study demonstrates, the starvation (causing quiescence) and reexposure to TPA (causing tumor-like growth and changes in differentiation) may offer the most direct and easiest access to genes involved in melanocytic growth and differentiation, which may also be important for neoplastic transformation and tumor promotion in the melanocytic system. It has been confirmed that TPA acts via the PKC pathway by mimicking the physiological ligand diacylglycerol (24, 25). The TPA pathway finally integrates with stress-induced pathways at AP-1 sites (26). The resulting changes regarding gene expression include enhanced expression of AP-1 components and increase the chance of transformation due to oncogene driven hypermutability, which has been identified as the cellular substrate of tumor promotion (27–29). Because PKC is the major cellular receptor for TPA (30), our data are consistent with the concept that the Lerk-5 gene is most probably controlled by signals transduced via the PKC pathway. Because Lerk-5 *eo ipso* represents an activating ligand of major eph-RTKs and is probably able to transduce signals due to receptor-like activity after phosphorylation of tyrosines of its cytoplasmic domain (*e.g.*, by platelet-derived growth factor-receptor tyrosine kinase-RTK), this study gives the first indirect evidence for a vivid cross-talk between these major signaling pathways.

We conclude that Lerk-5 mRNA overexpression and, probably, the consecutive activation of eph-RTKs in malignant melanomas and their metastases, either within the tumors (autocrine pathway) or between tumor cells and eph-RTK-expressing tissues, are probably very common functional phenomena in malignant melanomas. It possibly reflects or induces an increased potential of growth, tumorigenicity, and metastatic abilities in malignant melanomas. This makes the yet to be elucidated eph-RTK/Lerk signaling system a potential new source of diagnostic and prognostic markers, as well as a target for new therapies.

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